

Preliminary Amendment

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Applicants: Przybyla et al.

Serial No.: Unknown (Int'l. Application No. PCT/US99/31176)

Filed: On Even Date Herewith (Int'l. Filing Date: 12/29/99)

Title: RUBREDOXIN FUSION PROTEINS, PROTEIN EXPRESSION SYSTEM AND METHODS

Pat. No. 5,654,176); and the use of protease deficient strains of *E. coli* (Bibi et al., *Proc. Nat'l. Acad. Sci. (USA)* 90 :9209 (1993); D. Alexander et al., *Protein Exp. Purif.*, 3:204 (1992)). The importance of the cellular redox environment as a factor affecting folding and solubility of foreign proteins has been demonstrated through the use of the redox-active protein thioredoxin (12kD) as a fusion partner in expression systems (E.LaVallie et al., *Biotechnology* 11:187 (1993)) and through the synthesis of proteins in thioredoxin reductase (trx-) negative strains of *E. coli* (A. Derman et al., *Science* 262:1744 (1993)). These fusion systems have proven very useful, but the fusion products are sometimes difficult to follow during purification and there is still no assurance that any given protein will fold properly and/or become or remain soluble in any of the fusion systems in current use. Moreover, although the fusion partners maltose binding protein, glutathione-S-transferase and thioredoxin are typically derived from bacteria or protozoa, the existence of closely related mammalian and avian analogues of these fusion partners makes them unsuitable for use as anchor proteins for haptens in antibody production or in vaccines. Thus, continued development of new protein expression systems based on recombinant protein fusions with a stable carrier is necessary to advance the art of recombinant protein production.

Please replace the paragraph at page 25, line 27 to page 26, line 10, with the following rewritten paragraph. Per 37 C.F.R §1.121, this paragraph is also shown in Appendix A, with notations to indicate the changes made.

Rubredoxins from numerous different organisms have been isolated, and the amino acid sequences of various rubredoxins and the genes encoding various rubredoxins have been published. In this experiment the gene encoding rubredoxin from *D. vulgaris* St. Hildenborough was used (see Fig. 1;

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also Bruschi et al., *Adv. Exp. Med. Biol.* 74:57-67 (1976); Voordouw, *Gene* 67: 75-83 (1988)). The gene was amplified by polymerase chain reaction (PCR) from genomic DNA isolated from *D. vulgaris* using two primers and cloned into the expression vector pET24a (Novagen, Wisconsin) at the *Nde* I and *Bam*HI site. The pET-24a expression system utilizes the bacteriophage T7 promoter that serves as a binding site for T7 RNA polymerase and was incorporated into the chromosomal DNA of *E. coli* strain BL21 (DE3) (Novagen). T7 RNA polymerase is synthesized only upon the addition of isopropyl β -D-thiogalactoside (IPTG) to growing cultures since the gene for the T7 polymerase has been spliced into the chromosomal DNA of the *E. coli* host. The pET-24a plasmid also contains the gene for kanamycin resistance for selection of plasmid-containing colonies.

Please replace the paragraph at page 30, line 6 to line 24, with the following rewritten paragraph. Per 37 C.F.R §1.121, this paragraph is also shown in Appendix A, page AX, with notations to indicate the changes made.

Previous attempts to synthesize recombinant amyloid peptide in *E. coli* have resulted in the formation of inclusion bodies that required the use of guanidine thiocyanate for solubilization (B. Boyes et al., *J. Chromatog A.*, 691:337 (1995); Gardella et al., *Biochem. J.* 294:667-674 (1993)). A method for synthesizing this peptide as a recombinant fusion protein occurring in inclusion bodies was previously developed at Hoffman-La Roche (Döbeli, et al., *Biotechnology* 13:988-993 (1995)), but processing of their fusion to form pure monomeric A β ₁₋₄₂ is tedious in that it involves binding the fusion protein to a reverse-phase column followed by cyanogen bromide (CNBr) cleavage to remove the peptide from the fusion. Analysis of peptide purified with this method

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revealed formylation and carbamylation of the peptide as well as oxidation of Met-35. These alterations presumably occur as a result of CNBr cleavage of the peptide; Met-35 must be reduced by dimethylsulfoxide (DMSO) treatment in concentrated hydrochloric acid (HCl) before use. In this example, amyloid peptides were synthesized as fusions with rubredoxin in the hope of circumventing the difficulties of synthesizing homogeneous and consistently pure, monomeric peptides using existing methods. Recombinant synthesis as fusion proteins also allows more economical production of labeled peptides for use in continuing medical research efforts.

Please replace the paragraph at page 32, line 15 to page 33, line 23, with the following rewritten paragraph. Per 37 C.F.R §1.121, this paragraph is also shown in Appendix A, page AX, with notations to indicate the changes made.

Purification of β -amyloid 1-42 and 1-40

Following cleavage, the property of the $A\beta_{1-42}$ and $A\beta_{1-40}$ peptides to form sedimentable aggregates was used to concentrate and purify the peptide away from most of the rubredoxin moiety. But non-specific cleavage of both amyloid fusion proteins that occurs after Arginine-5 generated an additional peptide fragment that had to be separated from the intact peptides. The propensity of β -amyloid 1-42 to form aggregates and insoluble fibers poses a major problem in purifying this peptide (D. Burdick et al., *J. Biol. Chem.* 267:546 (1992), P. Sweeney et al., *Anal. Biochem.* 212:179 (1993)). Normal reverse phase chromatography is not a suitable method for purification. High temperature reverse phase chromatography using a Zorbax Stable Bond C18 column (McMod, PA) (B. Boyes et al., *J. Chromatog. A* 691:337 (1995)) at pH 2.5 (0.05%TFA) was thus attempted. Temperatures in the range of 80-85°C resulted in good